Protocol for Thawing, Expanding, Maintaining and Cryopreserving Adherent Cell Lines (October 2021)

Introduction

TZM-bl, 293S GnTI- and 293T/17 cell lines are integral reagents for many aspects of the neutralizing antibody assay. Thawing, maintaining, expanding and cryopreserving cells properly is crucial to ensure the viability and functionality of the cells throughout usage in the assays. When thawing and cryopreserving cells, it is important to remember to wear appropriate personal protective equipment (PPE) to minimize the risk of injury.

TZM-bl, 293S/GnTI- and 293T/17 are adherent cell lines. They must be disrupted from cell culture flasks in order for them to be added to the neutralization assay plate or used for transfection for the production of pseudoviruses or Infectious Molecular Clone (IMC) viruses. It is critical that this procedure be done carefully because the cells can easily be damaged if left exposed to the trypsin-EDTA for an extended period of time.

Definitions

ATCC: American Type Culture Collection

CO_2: Carbon Dioxide

DMSO: Dimethyl Sulfoxide

DPBS: Dulbecco's Phosphate Buffered Saline

FBS: Fetal Bovine Serum

Freezing media: 10% DMSO + 90% FBS

GM: Complete Growth Medium

LN_2: Liquid Nitrogen

PPE: Personal Protective Equipment

Reagents and Materials

Recommended vendors are listed. Unless otherwise specified, products of equal or better quality than the recommended ones can be used whenever necessary.

293T/17 Cells
Vendor: American Type Culture Collection (ATCC)
Catalog Number: ATCC CRL-11268

TZM-bl Cells
Vendor: NIH AIDS Reagent Program
Catalog Number: 8129

293S GnTI- Cells
Vendor: American Type Culture Collection (ATCC)
Catalog Number: ATCC CRL-3022

Complete Growth Medium (see Protocol: Reagent Preparation for Use in the Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells)

Dimethyl Sulfoxide (DMSO)
Vendor: Thermo Fisher Scientific

Fetal Bovine Serum (FBS)
Manufacturer: Nucleus Biologics

Dulbecco's Phosphate Buffered Saline (DBS)
Vendor: Thermo Fisher Scientific

Culture flasks with vented caps, sterile
T-25 flask
T-75 flask
T-175 flask
Manufacturer: Corning

Disposable pipettes, sterile, individually wrapped
1 ml pipettes
5 ml pipettes
10 ml pipettes
25 ml pipettes
50 ml pipettes
Manufacturer: Corning

Sterile Conical Tubes
15 ml
50 ml
Manufacturer: Corning

Sterile Microtubes
2.0 ml
Manufacturer: Sarstedt

70% ethanol solution
Manufacturer: generic

Instrumentation

Recommended manufacturers are listed. Unless otherwise specified, equipment of equal or better quality than the recommended ones can be used whenever necessary.

Biological Safety Cabinet
Manufacturer: Baker Co.

Incubator (37°C, 5% CO_2 standard requirements)
Manufacturer: Panasonic

Pipettor
PipetteAid XP
Manufacturer: Drummond Scientific Co.

Light Microscope
Manufacturer: Olympus

Hemacytomer or Automated Cell Counter (Countess, LUNA II)
Manufacturer: INCYTO or Invitrogen, Logos Biosystems (select appropriate)

Waterbath
Manufacturer: VWR International

Ultra Low Temperature Freezer (-70°C or below)
Manufacturer: Harris or Thermo Fisher Scientific

Cell Freezing Container
Manufacturer: BioCision

LN_2 Freezer and Tanks
Manufacturer: MVE Cryogenics

LN_2 Cryogenic Transfer Pan or Cool Rack
Manufacturer: Generic

4°C Refrigerator
Manufacturer: LABREPCO, Inc., Thermo Fisher Scientific

Protocol

1. Thawing cryopreserved cell lines

NOTE 1*:* The thawing procedure is stressful to frozen cells, and using good technique and working quickly ensures that a high proportion of the cells survive the procedure.

NOTE 2*:* Be sure to wear a full-face shield or goggles during the handling of frozen specimens. Also, frozen specimens should be placed in a closed vented container with dry ice during transport from the freezer to the laboratory.

1.1. Warm Complete Growth Medium (GM) in 37°C waterbath to 20°C-37°C.

1.2. Transfer cryovials containing frozen cells from liquid nitrogen to a 37°C water bath. Use a cryotransfer pan or cool rack as necessary if thawing cells within a biological safety cabinet.

1.3. If liquid nitrogen has potentially seeped into the cryovial, loosen the cap slightly to allow the nitrogen to escape during thawing.

1.4. Thaw the cryovial on the surface of the water bath with an occasional gentle "flick" during thawing. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Do not leave the cryovial unattended during the thawing process. (It is important for cell viability that the cells are thawed and processed quickly – thawing only takes a few seconds).

1.5. Dry off the outside of the cryovials and wipe with a 70% ethanol solution before opening the vial to prevent contamination.

1.6. Depending on laboratory specific preferences, follow the procedure outlined in 1.7 or 1.8.

NOTE 3: Before thawing, pre-label a T-75 or T-25 sterile, vent cap culture flask with cell type, thaw date, cell count labeled on frozen vial and passage number.

1.7. Transfer the contents of one vial of thawed cells to a T-75 culture flask containing 30 ml of GM.

1.8. Transfer the contents of one vial of thawed cells to a 15 ml conical tube and slowly add 10ml GM. Gently spin the cells until a pellet is formed. Aspirate media/DMSO, resuspend in 10 ml fresh GM and repeat the spin. Aspirate off the media, resuspend in 10 ml fresh GM and then add to a T-25 culture flask.

NOTE 4: It is important to dilute the cyroprotectant DMSO present in the cryovial at least 30-fold to avoid cell toxicity.

NOTE 5: Wear proper personal protective equipment when working with DMSO, as this material readily penetrates skin and has the potential to be harmful.

1.9. Incubate the cells at 37°C/5% CO_2 overnight, keeping the flasks in a horizontal position (TZM-bl, 293T/17 and 293S/GnTI-).

1.10. The next day, remove the medium and replace with 15 ml of fresh GM. Change the medium every 2-3 days until the cells are confluent.

2. Maintenance and Expansion of TZM-bl Cells

NOTE 6: TZM-bl cells are an adherent cell line that is maintained in T-75 culture flasks. Cell monolayers are disrupted and removed by treatment with Trypsin-EDTA at confluency when splitting cells for routine maintenance and when preparing cells for assays. Cells may be used up to passage 60 in culture or 5 months in culture, whichever comes first.

NOTE 7: Flasks for cells in culture should be labeled with cell type, passage number, seed cell count, date of passage and optimal day of usage.

2.1. Warm GM in 37°C waterbath to 20°C-37°C.

2.2. Remove the culture medium and eliminate residual serum by rinsing monolayers with 6 ml of sterile DBS.

2.3. Slowly add 2.5 ml of 0.25% Trypsin-EDTA solution to cover the cell monolayer.

2.4. Incubate at room temperature for 30-45 seconds.

2.5. Remove the Trypsin-EDTA solution and incubate at 37°C for 3-4 minutes. Do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach.

2.6. Add 10 ml of GM and mix the cells by gentle pipette action. Count cells via hemacytometer or other cell counting device.

2.7. Seed new T-75 culture flasks with the appropriate number of cells depending on when cells will be needed for the neutralizing antibody assay, as indicated in Table 1 below. Cultures are incubated at 37C in a 5% CO_2/95% air environment. Cells should be split again or used upon confluency.

NOTE 8: The amount of GM+cells should equal 15 ml in a T-75 flask.

Table 1.

Number of viable cells seeded in new flask	Days in incubator before confluency
0.5 x 10^6	5
1.0 x 10^6	4
2.0 x 10^6	3
4.0 x 10^6	2

2.8. Cells should be tested for *Mycoplasma* on a predetermined basis.

3. Maintenance and Expansion of 293T/17 and 293S/GnTI- cells

NOTE 9: 293T/17 and 293S/GnTI- are adherent cell lines that are maintained in T-75 culture flasks. Cell monolayers are disrupted and removed by treatment with Trypsin-EDTA at confluency. Cells may be used up to passage 60 in culture or 5 months in culture, whichever comes first.

NOTE 10: Flasks for cells in culture should be labeled with cell type, passage number, seed cell count, date of passage and optimal day of usage.

3.1. Warm GM in 37°C waterbath to 20°C-37°C.

3.2. Remove the culture medium and eliminate residual serum by gently rinsing the monolayers with 6 ml of sterile DBS.

3.3. Slowly add 2.5ml of a 0.25% Trypsin-EDTA solution to cover the cell monolayer. Immediately remove the Trypsin-EDTA solution and incubate at room temperature for 30-60 seconds. Do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach.

3.4. Add 10 ml of GM and suspend the cells by gentle pipette action. Count the cells via hemacytometer or other counting device.

3.5. Seed new T-75 culture flasks with the appropriate number of cells, as indicated in Table 2 below. Cultures are incubated at 37°C in a 5% CO_2/95% air environment. Cells should be split again or used upon confluency.

NOTE 11: The amount of GM+cells should equal 15 ml in a T-75 flask.

Table 2.

Number of viable cells seeded in new flask	Days in incubator before confluency
2.0 x 10^6	4
4.0 x 10^6	3

3.6. Cells may be expanded using T-175 flasks. Seed 9.0 x 10^6 viable cells into a final volume of 45 ml GM and incubate for 2 days to achieve confluency.

3.7. Cell cultures should be tested for *Mycoplasma* contamination on a predetermined basis.

4. Cryopreservation of TZM-bl, 293S/GnTI- and 293T/17 cells

NOTE 12: Use the same cryopreservation procedure for all cells.

4.1. After detaching cells from flask as described in section 2.0 for TZM-bl cells, or section 3.0 for 293S/GnTI- and 293T/17 cells, resuspend cells in 5-10 ml of GM and count cells.

4.2. Prepare an adequate number of cryogenic vials to aliquot cells:
TZM-bl, 3-3.5 x 10^6 cells per vial;
293T/17 and 293S/GnTI-, 5-10 x 10^6 cells per vial.

4.3. Prepare an appropriate volume of freezing media, consisting of 10% DMSO and 90% FBS. DMSO is added to prevent the formation of ice crystals during the freezing process.

4.4. Centrifuge cells at ~ 200 x g for 5 min to pellet cells. Decant the supernatant.

4.5. Resuspend cells in freezing medium to a concentration of 3 x 10^6 cells/ml (TZM-bl) or 5-10 x 10^6 cells/ml (293T/17 or 293S/GnTI-).

4.6. Aliquot 1 ml of cells into labeled cryogenic storage vials. Start the freezing procedure within 5 min.

4.7. Cells should be frozen slowly at 1°C/min. This can be achieved by placing vials in a Cryo 1°C CoolCell Freezer Container (or equivalent).

4.7.1. If a cell freezing container is utilized, it should be stored in a -70°C to -90°C freezer overnight prior to transferring vials to permanent storage in a liquid nitrogen tank.

NOTE 13: The laboratory maintains a frozen Archive Master Stock and Working Master Stock of each cell line. When a Working Stock is exhausted, a new batch should be prepared using the Archive Master Stock. It is advisable to keep Master Stock and Working Stock for each cell line, in more than one freezer for redundancy, in case of a freezer thaw/disaster.

NOTE 14: Use laboratory specific tracking log or notebook for recording pertinent information related to freezing and/or thawing cells.

References

1. Protocol for Reagent Preparation of Use in the Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells

2. Protocol for Preparation of Cells for Detection of Mycoplasma Species